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Mutagenesis Computer Experiments in Pentameric Ligand-Gated Ion Channels: the Role of Simulation Tools with Different Resolution

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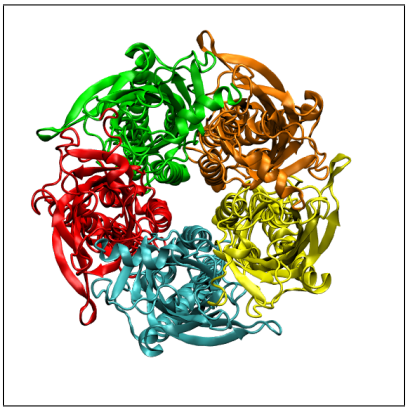
Abstract

Pentameric ligand-gated ion channels are an important class of widely expressed membrane neuroreceptors, which play a crucial role in fast synaptic communications and are involved in several neurological conditions. They are activated by the binding of neurotransmitters, which trigger the transmission of an electrical signal via facilitated ion-flux. They can also be activated, inhibited or modulated by a number of drugs. Mutagenesis electrophysiology experiments, with natural or unnatural amino acids, have provided a large body of functional data that, together with emerging structural information from X-ray spectroscopy and cryo-electron microscopy, are helping unravel the complex working mechanisms of these neuroreceptors. Computer simulations are complementing these mutagenesis experiments, with insights at various levels of accuracy and resolution. Here we review how a selection of computational tools, including first principles methods, classical molecular dynamics and enhanced sampling techniques, are contributing to construct a picture of how pentameric ligand-gated ion channels function and can be pharmacologically targeted to treat the disorders they are responsible for.

Graphical TOC Entry



Keywords

Pentameric ligand-gated ion channels, mutagenesis electrophysiology experiments, first principles methods, molecular dynamics, enhanced sampling methods, metadynamics.

Introduction

Pentameric ligand-gated ion channels (pLGICs) are membrane receptors, located at synapses, which convert chemical signals from presynaptically released neurotransmitters into postsynaptic electrical signals. They mediate fast synaptic communication, which plays a crucial role in processes like memory, learning and sensory perception. They are widely expressed in multicellular animals, as well as in bacteria. They are associated with a variety of neurological conditions where neuronal communication is impaired, from Alzheimer's and Parkinson's disease to addiction, attention deficit disorder and schizophrenia. They are important target sites for drugs, anaesthetics and, in invertebrates, insecticides.[1–5]

pLGICs share common structural features and some degree of sequence homology: a model of a prototypical example, the serotonin (5-hydroxytryptamine, 5-HT) type 3 receptor (5-HT_3R), is shown in Fig. 1. They are composed of five identical (homo-pentameric LGICs) or different (hetero-pentameric LGICs) subunits arranged in a cylindrical shape around an ion permeable pore. Each subunit consists of: (i) an extracellular domain (ECD), mostly made of β-sheets, with the β strands progressively labelled from β1; (ii) a transmembrane domain (TMD) embedded in the cell membrane, composed by four interconnected helices labelled as M1, M2, M3 and M4; and (iii), in some cases, an intracellular domain (ICD). The receptors are activated by the binding of neurotransmitters at the interface between subunits in the ECD, as shown in Fig. 2 for an exemplary case (GABA binding to the insect resistance to dieldrin (RDL) receptor). It is not clear how many neurotransmitters are needed for optimal activation, but experimental evidence suggests two or three.[1,6] Neurotransmitter binding induces conformational changes which result in the opening (gating) of the ion channels: ions can then pass through the membrane transmitting the electric signal between neurons. Ion flow produces either a depolarisation response, for excitatory anionic receptors, or a hyperpolarisation one, for inhibitory cationic receptors. The activity stops when the channel closes due to the absence of neurotransmitters or assumes a desensitized non-conductive state with ligands still bound.

Examples of pLGICs include the nicotinic acetylcholine receptor (nAChR),[7] $GABA_A$ and $GABA_C$ receptors, glycine receptors (GlyR), the serotonin 5-HT_3R[6] shown in Fig. 1, the eukariotic glutamate receptor GluCl, and the bacterial receptors *Erwinia Chrysanthemi* and *Gloeobacter Violaceus* ligand-gated ion channels (ELIC and GLIC respectively). Some of these pLGICs (e.g. nAChR, GABA receptor, GlyR and 5-HT_3R) are grouped into the Cys-loop superfamily, characterized by a disulphide bridge that joins two cysteines 13 residues apart, forming the characteristic Cys-loop in the ECD N-terminal. Besides specific neurotransmitters (e.g. acetylcholine, GABA and serotonin), these receptors can bind other natural (e.g. nicotine for nAChR) or artificially-designed ligands, both in the orthosteric binding sites or allosterically,[8] which can activate, potentiate or impair channel function. For example, granisetron is an antiemetic drug that blocks the activity of 5-HT_3R, which is linked to the vomiting centre,[9] while benzodiazepines enhance the inhibitory effects of GABA in the $GABA_A$ receptors, resulting in sedative, anxiolytic, anticonvulsant and muscle relaxant effects.

Despite pLGIC medical and pharmacological importance, how the binding information is passed from the ECD to the TMD tens of Angstroms away, to induce the opening of the channel, is still far from being fully understood. As evident in Fig. 1 for 5-HT_3R, these two domains are topological distinct, but can communicate through a number of crucial flexible interface loops, including the ECD Cys-loop and the TMD M2-M3 loop connecting helices M2, which lines the channel, and M3.

Difficulties in fully unravelling the working mechanisms of pLGICs arise from their intrinsic complexity as well as that of the environment they are embedded in, and from the limited available structural information, due to the challenges in crystallising and resolving these membrane receptors. Some progress was first achieved thanks to the X-ray structure of the acetylcholine binding protein (AChBP),[10] a globular snail protein similar to the ECD of nAChR, which thus lacks the TMD, and of a medium resolution electron microscopy structure of nAChR from the electric ray *Torpedo Marmorata*.[11] To achieve crystallisation,

in many cases intracellular loops are either removed or replaced by short linkers similar to prokaryotic Cys-loop receptors, so ICD information is often missing or incomplete, although their role can be important, e.g. for interacting with scaffold proteins supporting synapse formation. Given the modular nature of these receptors, single subunits or domains can also be studied separately, although with some limitations. Recently, however, some fairly complete and full length structures with good resolution in different functional states have become available from X-ray spectroscopy and cryo-electron microscopy.[12–26] More are likely to be determined in the near future, providing unprecedented information and opportunities for modelling.

Many chemical-scale insights into the relationship between structure and function of pLGICs have been obtained by mutagenesis electrophysiology experiments, whose main goal is to assess how mutations impact on receptor structure and function. Mutations can be realized with natural amino acids or artificially designed compounds, to increase diversity and tailor experiments to probe e.g. the role of specific bonds. A powerful tool for incorporating unnatural amino acids into membrane proteins is the *in vivo* nonsense suppression method, which, through a combination of organic synthesis and molecular biology, prepares novel aminoacyl tRNAs to deliver unnatural amino acids to the ribosome,[27] often using *Xenopus* oocytes as expression tools. The function of mutated receptors can then be probed by electrophysiology measurements, which are often quantified by the effective concentration half (EC_{50}, a functional measurement of the ligand concentration which results in a half of the maximum response). Mutated receptors with higher EC_{50} either bind ligands with lower affinity with respect to the wild-type and/or are less efficient at channel opening than those with lower EC_{50}.[28–33] Moreover, patch clamp techniques allow recording of single-channel kinetics in real time.[34]

The experimental response is, however, macroscopic: its interpretation can be supported and improved by computer modelling, which can mimic experiments and provide otherwise inaccessible details at the molecular level. Here, we review our work, focused on using a

series of simulation tools of different resolution to assess the effects of mutations in pLGICs. We also give an overview of selected work by other groups focussed on mutations in pLGICs so to provide a more general picture.

Atomistic models of pLGICs can be built by homology, or be based on X-ray and cryo-electron microscopy structures, when available. Given the intrinsic complexity of pLGICs, simulation techniques of different resolution and accuracy, used individually or in combination, are needed to elucidate different aspects of pLGICs working mechanisms. Most commonly, these include first principles methods (specifically density functional theory, DFT), classical molecular dynamics (MD) with all-atom force-fields, and enhanced sampling methods (in particular metadynamics): they will be expanded in the following.

Although additional techniques such as Monte Carlo simulations,[35–37] coarse-grained methods,[38,39] umbrella sampling,[40,41] steered MD,[39] the string method[42] and tunnelling calculations[43] are available and have been successfully used to investigate ion channels, a full description of these is beyond the scope of this review.

The behavior of pLGICs is extremely complex and an ensemble of multiple related conformations may be needed to describe a physiological state. While more experimental structural information is progressively becoming available for pLGICs in different functional states, it is extremely challenging to unambiguously match experimental structures to functional states (open, closed, desensitised, inhibited etc..), due to a variety of factors, including limited resolution, influence of detergent and/or nano-bodies, crystal packing and receptor engineering.[26] Due to the still limited availability of structural models, the complexity and size of the systems and the relatively short time scales that can be simulated, computational works like those reviewed here are often limited to explore only one functional state of the receptor of interest. Therefore such studies do not provide information on how the equilibrium between different states is altered by mutations, which would be an important topic to address in the future. Nonetheless, they prove very useful in highlighting dynamical and structural differences between wild-type and mutated receptors.

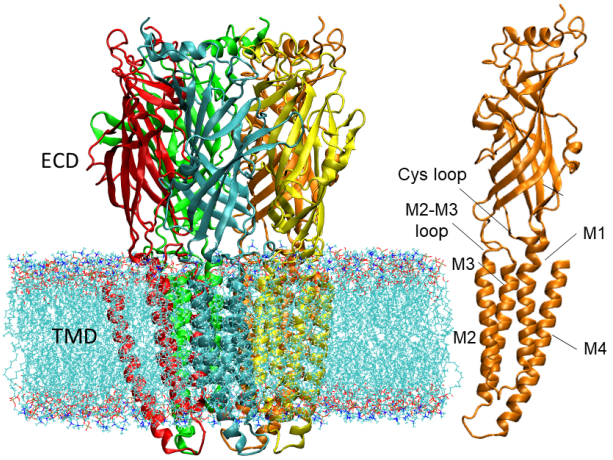


Figure 1: Left: Model of a prototypical pLGIC, the mouse homo-pentameric 5-HT_3 receptor, based on the X-ray structure of Ref.[17] The incompletely resolved intracellular domain has been removed, and the M3 and M4 helices have been linked with a short loop. The model is embedded in a POPC lipid bilayer mimicking the cell membrane. Right: a single subunit with relevant structures labelled.

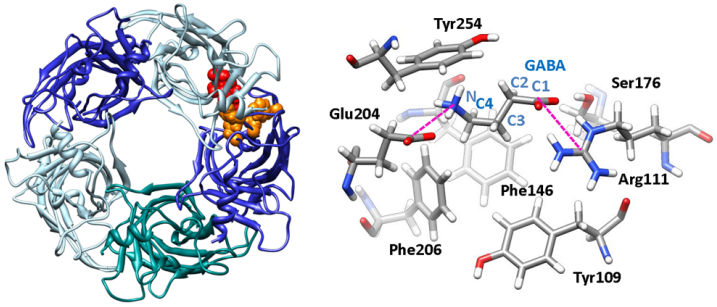


Figure 2: An exemplary orthosteric binding site in a pLGIC. Left: Top view of model of the ECD of the RDL receptor, with residues in the orthosteric binding site in red and orange. Right: GABA in the binding site, including aromatic residues which form a "cage" around the GABA amine group. [Adapted from Ref.[44]]

First Principles Methods

First principles methods, in particular based on DFT which offers a favourable ratio between accuracy and computational cost, can provide precise insights into the electronic structure effects produced by mutations. However, because of their computational cost, their application is limited to small models, e.g. of the binding site of pLGICs, or to supporting calculations to parametrise force-fields, for example for non-standard ligands and for unnatural amino acids, to be used in extended classical MD or enhanced sampling simulations of mutated receptors.

Neurotransmitters usually bind in zwitterionic form (with moieties of opposite charges, e.g. GABA) or carry a positive charge (e.g. serotonin). Besides being involved in hydrogen bonds and salt-bridges (as well as their negative counterparts), positively charged groups can interact with the aromatic rings of amino acids like tyrosine, phenylalanine or tryptophan through cation-π interactions.[45] A cage of aromatic amino acids is thought to often enclose the positively charged part of the ligand in the orthosteric binding site of pLGICs as shown in Fig. 2. Fluorination of the aromatic rings of these amino acids decreases aromaticity, weakening cation-π interactions as well as hydrogen bonds: it is a powerful strategy to obtain information on the relevance of such interactions to ligand binding. These are electronic structure effects that can be effectively captured by first principles calculations.

As an example, Fig. 3 shows electronic structure data obtained with DFT (with the PBE exchange and correlation functional, norm-conserving Martins-Troullier pseudopotentials and a plane wave basis set with a cutoff of 70 Ry) for a reduced model of the binding site of serotonin in the 5-HT_3 receptor, that we built to assess the effects of mutating specific tyrosines. The latter were modified, as in experiments, by substituting the hydroxyl group and hydrogen atoms in the aromatic side chain with fluorine. The data in Fig. 3 compares the wild-type with the extreme case of Tyr mutated into F_5-Phe, where the hydroxyl group and all the four hydrogens attached to the aromatic ring are substituted by fluorine atoms, resulting in a total loss of aromaticity; the signature of the typical negative

charge ring totally disappeared from the electrostatic potential mapped onto an electronic density isosurface upon fluorination as evident in Fig. 3. The decrease in hydrogen bond strength is demonstrated by the rearrangement of the electronic density upon formation of the hydrogen bond between serotonin and either Tyr or F_5-Phe; the region affected lies in between hydrogen bond donor and acceptor with evident oscillations (charge gain/depletion) for Tyr but no significant charge-transfer for F_5-Phe, consistent with the hypothesis of a substantially weaker bond. The electron localization functions (ELFs), which indicate where electrons are maximally localized, show that in the tyrosine the oxygen lone pair is oriented in the direction of the hydrogen bond with serotonin, while the fluorine lone pairs in F_5-Phe tightly surround the atoms. In fact, organic fluorine hardly forms hydrogen bonds if better acceptors, like oxygen or nitrogen, are present; it acts as a weak hydrogen bond acceptor, like in the calculations shown, in case of no alternatives. These calculations, although on very simple models, helped rationalize experimental results where mutations of crucial tyrosine residues with F_5-Phe and other fluorinated unnatural amino acids produced substantially increased EC_{50} or no response, due to the role of such tyrosines in alleged cation-π or hydrogen bond interactions in the serotonin binding site in 5-HT_3R.[28]

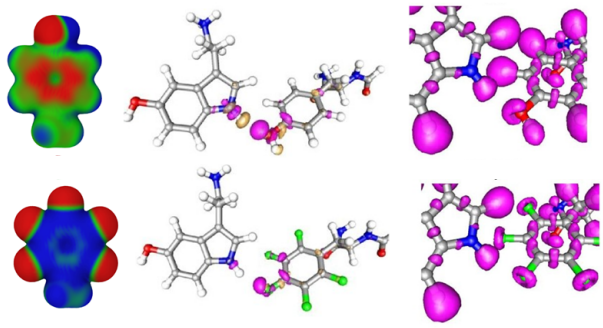


Figure 3: Left panels: Electrostatic potential (blue positive, red negative, green neutral) mapped onto an electronic density isosurface of 0.01 $e/Å^3$ of the side chain of tyrosine (top) and F_5-Phe (bottom). Central panels: Electronic density differences due to the hydrogen bond formation between serotonin and either tyrosine (top) or F_5-Phe (bottom) in a reduced model of the 5-HT_3R binding site. Purple isosurfaces correspond to electronic charge gain and orange isosurfaces to electronic charge depletion. Right panels: Electron localisation function isosurfaces (0.85) for serotonin interacting with tyrosine (top) or F5-Phe (bottom). [Adapted from Ref.[46]]

The previously mentioned structural motif of an aromatic cage surrounding a cation interacting with multiple aromatic residues, proposed for the binding site of pLGICs, was also studied by DFT calculations with the M06 exchange and correlation functional,[47] together with the effects of progressive ring fluorination to support the strategy used in *vivo* mutagenesis experiments to probe cation-π interactions.

As shown in Fig. 3, the analysis of the electrostatic potential of the mutated amino acids mapped onto an electron density isosurface is a useful tool for estimating the efficiency of an aromatic residue to perform cation-π interactions. In general, the stronger is the negative electrostatic potential in the ring, the stronger is the interaction: this is reflected in the calculated binding energies.[48,49] The effects of mutations in cation-π interactions can be qualitatively explained in simple electrostatic terms by assuming that the electrostatic potential calculated at points located above the centre of the ring is able to predict the strength of the interaction. However, additional effects have been suggested as important for the description of such a complex interaction, including inductive effects over π resonance[49] and the effects of mutation on the polarization of the π-ring.[50] It has also been shown that that the effect of mutations can be attributed to direct through-space interactions with the substituents.[51,52] An alternative description of cation-π interaction is based on the estimate of the interaction between the ligand monopole and the aromatic molecular quadrupole moment;[53] however, the description in terms of electric multipoles is only valid for well-separated charge distributions.[54]

Within the study of pLGICs, as well as other biomolecules, first principles calculations play also a very important role in the development of force-field parametrisations for unconventional ligands and unnatural amino acids to use for large simulations. For example, we used DFT calculations (using PBE gradient-corrected exchange-correlation functional, norm-conserving Martins-Troulliers pseudopotentials and a plane-wave basis set with a 70 Ry cutoff) to parametrise a series of proline analogues,[55] which were used in experiments to mutate a specific proline in the M2-M3 loop in 5-HT_3R.[56] This loop, at the interface

between the ECD and the TMD, is believed to play a crucial role in transmitting the binding information to the channel. The mutants were chosen to favour or disfavour *trans-cis* isomerisation of the prolyl peptide bond and involved modification of the number of atoms in the Pro ring (Pip, Aze), its fluorination (c-4F-Pro, t-4F-Pro) and the addition of chemical groups (Dmp, 2-Me-Pro, Tbp, 3-Me-Pro).[56] Partial charges were calculated from first principles and the parameters describing the relevant torsion for isomerisation were optimised to reproduce DFT calculations. The force-field parameters were then used for enhanced sampling calculations, as described in the relevant section.[55]

In summary, first principles calculations for pLGICs currently play a useful, but rather limited supporting role. Given the size and the timescale involved in the activation processes, these methods cannot be employed yet for fully modelling the neuroreceptors; however, they can complement coarser (and less accurate) levels of description applied to complex structures, and support the interpretation of experiments when subtle electronic effects are involved. They can also be employed within hybrid QM/MM schemes, where only a portion of the system is treated by quantum mechanical (QM) methods and the rest with classical force-fields (MM, molecular mechanics), a combination successfully used for several biological systems in static or dynamical calculations.[57–60] Sampling time is however dominated by the cost of the QM part. With the increasing availability of experimental structures to build reliable models, efficient linear-scaling algorithms for electronic structure calculations and computer power, applying QM/MM schemes to pLGICs may become more common, especially in cases where electronic structure effects, which cannot be described by force-fields, need to be captured within the receptors.

Classical Molecular Dynamics

Classical molecular dynamics simulations play an important role in providing information at the molecular level for pLGICs at ambient or physiological conditions. Starting from available

experimental structures or from homology models, the dynamics of fairly complete pLGICs (e.g. in Refs.[61–63]) or portions of them, like the ECD or the TMD, (e.g. in Refs.[44,64–67]) can be studied for significant times. The availability of powerful computers, MD software packages, homology modelling software and, more recently, good resolution structures, provides the opportunity to set up interesting simulations, accounting for the environment and mimics of the membrane. Ligand-docking, assisted by experimental information from mutagenesis experiments, helps locate neurotransmitters or relevant ligands in potential binding pockets, generating initial configurations for MD simulations. Once these are carried out, information on the network of interactions formed by the ligand can be extracted and compared with experimental evidence. Mutations can be easily included in the models, and MD simulations can be repeated to reproduce the work of mutagenesis experiments.[64,65]

For example, to complement single-channel experiments and kinetic calculations, we designed a set of MD simulations to mimic mutagenesis experiments in the α7 nicotinic acetylcholine receptor with the goal to understand the role of a specific proline (Pro180) in the middle of the ECD β9 strand during desensitisation, i.e. the process involving progressive reduction of the flux of ions when the ligands are present for prolonged times.[65] The simulations used a minimal homology model built on the AChBP template, with two adjacent subunits and the AMBER2003 force-field. Pro180 was mutated with Thr, Ser, Tyr, Asp, Gly and Phe, and simulated for a few ns at ambient temperature and pressure. In all cases the residues at position 180 in the β9 strand formed double the number of hydrogen bonds with the adjacent β10 strand, as shown in Fig. 4 together with typical quantities that can be calculated from MD trajectories related to residue fluctuations, bond length distributions and representative snapshots. The disruption of these interactions when proline is present at position 180, due to its cyclic structure, correlates with faster desensitisation times recorded experimentally, therefore linking molecular level details to macroscopically recorded effects and providing insights into how this residue facilitates rapid desensitisation while its mutants do not.

We also performed MD simulations (at 310 K and 1 atm for a few ns with the AMBER2003 force-field) for a series of mutants of the ECD of the $GABA_C$ receptor, a type of $GABA_A$ receptor mostly located in retinal neurons with a potential role in retinal signaling and disease like macular degeneration.[64] The carboxylate group of GABA in zwitterionic form is likely to bind to a positively charged residue, typically an arginine[44,64,68] (here Arg104, in the example of Fig. 2 Arg111), while its amine group is likely to be involved in cation-π interactions within a cage of aromatic residues. Mutating Arg104 with neutral (Ala), negatively charged (Glu) or positively charged but less bulky (Lys) residues experimentally resulted in a very large increase of EC_{50} or insensitivity up to large GABA concentrations,[30] which correlates well with the MD data. These in fact revealed, at the atomistic level, the persistence of hydrogen bonds between Arg104 and GABA for much of the MD simulation in the wild-type, but extremely infrequent interactions when this residue was mutated to Lys and no bonds at all when mutated to Ala or Glu.[64]

Many other MD simulations on a variety of pLGICs have been performed to complement experiments and help rationalise the effects of mutations; they are often found in the literature in joint experimental and computational papers and they have various aims, such as understanding the action of particular drugs or the role of specific residues in the receptors (see e.g. Refs.[21,69–79]). Much work has been done on the bacterial channels ELIC and GLIC due to the availability of structural information providing reliable initial templates for MD simulations.

A recent example investigated GLIC in complex with the surgical agent propofol, which is used for sedation.[78] μs-long MD simulations at ambient conditions and electrophysiology experiments were carried out to understand the effects of mutations in a transmembrane binding cavity, specifically acting on residue Met205 within the M1 transmembrane helix (shown in Fig. 5 on the left), and obtaining mutants potentiated by propofol. Anaesthetic potentiation was associated with enhanced electrostatic interactions in membrane-accessible sites, while inhibitory effects were linked to solvent-accessible sites. MD simulations were able

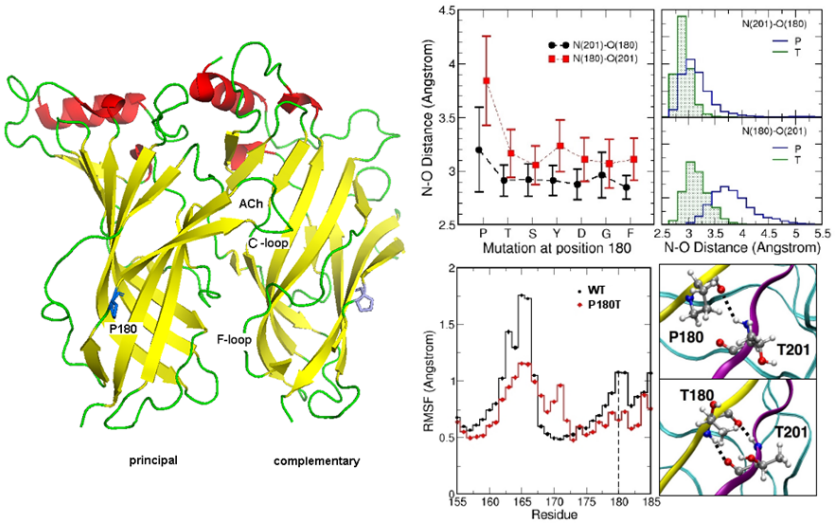


Figure 4: Left: Model of $\alpha 7$ nAChR with Pro180 labelled. Right: Hydrogen bonds at position 180 during MD simulations. Top: Average distance and standard deviation between the amine nitrogen of the residue at position 180 in the $\beta 9$ strand and the carboxylic oxygen of Thr201 (red line) in the $\beta 10$ strand, and between the amine nitrogen of Thr201 and the carboxylic oxygen of the residue at position 180 (black line) for the wild-type Pro and investigated mutations. Distributions of the N-O distances for the wild-type receptor (blue line) and Pro180Thr-mutated receptor (green line). Bottom: Root mean square fluctuations (RMSF) with respect to the average structure of the residues around the $\beta 8$-$\beta 9$ loop and position 180. Two MD snapshots showing the hydrogen bonds formed by the residue at position 180 and Thr201 for the wild-type receptor and the Pro180Thr mutant. [Adapted from Ref.[65]]

to assess the altered dynamics of the binding cavity volume upon mutations and the ligand penetration, together with the evaluation of hydrogen bonds with the M1 helix. Other studies based on mutagenesis electrophysiology experiments and related MD simulations were performed for GLIC in complex with propofol and desflurane,[72] desflurane and chlorophorm,[75] halothane and thiopental,[71] and for ELIC with isoflurane,[76] with the goal to assess the role of specific residues in the mechanisms of action of anaesthetics in pLGICs.

Besides anaesthetics, the interaction of pLGICs with neurotransmitters[74,79] and a variety of drugs[21] have been investigated with the support of MD simulations. For example, a co-crystal structure of ELIC in complex with chlorpromazine, an antipsychotic medication, was determined (shown in the centre of Fig. 5).[21] The chlorpromazine binding site was identified behind the ECD β8-β9 loop, whose residues were in turn mutated to cysteine. It was demonstrated that chlorpromazine binding at this loop is responsible for receptor inhibition; 500 ns MD simulations of the chlorpromazine-bound and apo receptor (at 310 K and 1 bar, with the AMBER99SB-ildn force-field) showed a ligand-induced conformational change of the β8-β9 loop, resulting in an allosteric binding site. The action of alcohol on pLGICs (specifically GLIC) and on specific alcohol-sensitive mutants was also investigated with the support of MD.[73,80]

The X-ray structure of the open form of GLIC highlighted the contribution of rings of hydroxylated residues facing the channel (Ser6′ and Thr2′) in the TMD;[12] single-channel electrophysiology showed that the side chain of Ser6′ (shown in Fig. 5, on the right) is crucial for ion translocation, and 200 ns-long MD simulations, at 310 K, provided details of the effects of these residues on the hydration of the pore. Mutations in the TMD can also be used to enlarge the pore size or stabilise the channel open/conductive or closed/non-conductive state: channel size and properties can be probed by MD simulations, which in turn may suggest mutations that would determine certain effects.[69,70,81]

While some of the reviewed studies may seem very specific and focused on the role of particular residues, because of structural similarities of pLGICs and the conservation

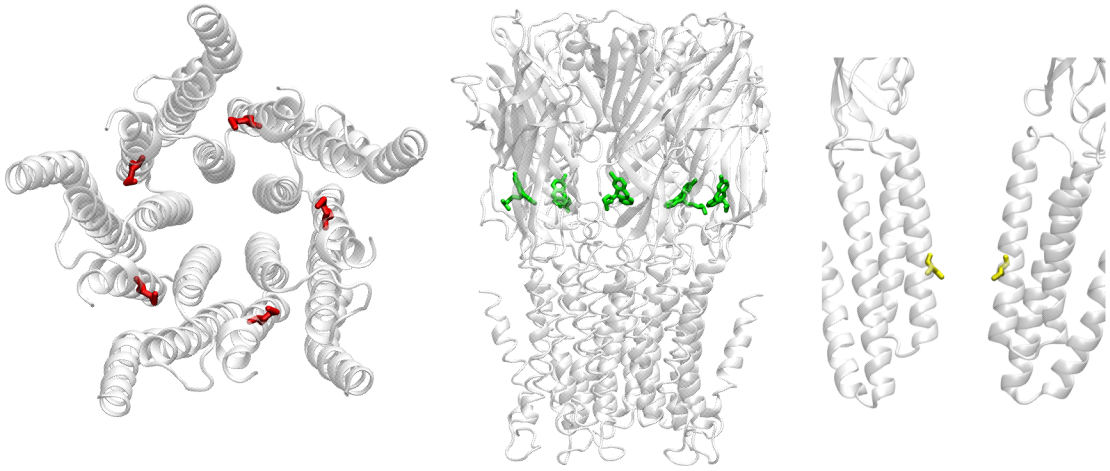


Figure 5: Left: top view of the transmembrane domain of wild-type GLIC (PDB entry: 3P50) with Met205 in red investigated in Ref.[78] Centre: side view of the X-ray structure of ELIC in complex with chlorpromazine (in green) (PDB entry: 5LG3) used in the work of Ref.[21] Right: zoomed view of two subunits of GLIC (PDB entry: 4HFI) with Ser6′ highlighted in yellow, used in Ref.[12]

of a number of key residues, the information gained can often be extrapolated to other pLGICs. For example, the mutation of Met205 studied in Ref.[78] is of general relevance due to its proximity to Pro204, which is a conserved transmembrane residue among pLGICs and whose hydrogen bond disrupting effect may be relevant for gating. The findings in Ref.[21] were discussed in a general context with a comparison of the allosteric binding sites of the closed, open and desensitised states of Cys-loop receptors. Results for TMD Ser6′ in GLIC[21] were supported by previous mutational studies of Ser6′ and Thr2′ in nAChR.[82]

In summary, MD is a very powerful tool to extract atomistic information from a pLGIC model and assess the effects of mutations at finite temperature. The reviewed selected examples cover a variety of cases, including the investigation of orthosteric and allosteric binding sites for neurotransmitters and drugs, specific residues in the ECD, the TMD and the pore. However, MD is limited to timescales (typically up to μs) sufficient to sample local free energy minima. In most cases, MD is unable to access, with affordable computational resources, conformational states separated by energy barriers, as well as to describe activated processes like ligand binding (from the solvent) and unbinding, molecular switches,

gating mechanisms etc., which occur on much longer timescales. Strategies to overcome this limitation involve either the use of simplified coarse-grained models or exploiting enhanced sampling techniques, designed to accelerate rare events, as described in the next section.

Enhanced Sampling Methods

Enhanced sampling methods aim at overcoming the inadequacies of MD simulations which are due to limited sampling of rough free energy landscapes with local minima separated by relatively large energy barriers, as typical of many biological processes.[83]

Among the many available enhanced sampling methods, metadynamics stands out for its relative simplicity and successful applications in different fields, from condensed matter physics, chemistry, materials science and biology. It allows to accelerate rare events and calculate the free energy of complex polyatomic systems.[84,85] Metadynamics is based on a dimensional reduction and the identification of a small set of slowly varying variables (collective variables, CVs) that describe the process of interest and, in particular, the minimal free energy conformations. The molecular dynamics of the system is enhanced by adding a history-dependent potential, built as a sum of Gaussians located at points already visited in the selected CV space. In practice, this repulsive potential discourages the system to revisit places already visited, encouraging it to escape local minima and sample the whole CVs space. When the sampling is complete, the history-dependent potential provides a cast of the underlying free energy landscape as a function of the chosen CVs. An efficient metadynamics simulation relies on a smart choice of a few CVs, able to capture the important details of the process of interest. Metadynamics has been successfully used by us and many others to accelerate rare events and explore free energies in small and large biomolecules, including systems with mutations (see e.g. Refs.[55,62,68,86–96]). Here we focus on its applications to investigate mutations in pLGICs.

We used metadynamics to investigate the disruption of neurotransmitter binding in a

GABA receptor when crucial residues were mutated.[68] For this task, we considered the RDL receptor, an insect receptor activated by GABA, which is linked to insecticide resistance and is a potential target for insecticide design.[97–99] The RDL receptor had been previously studied by us computationally, by homology modelling to test different recipes and select a reliable structure, ligand-protein docking guided by experimental information and MD,[44] and by mutagenesis electrophysiology experiments.[32,66] While these calculations, with the support of experiments, provide information on the reliability of the atomistic model and on the network of potential interactions that GABA may form in the receptor, as shown in Fig.2, they depend on the reliability of the initial docked pose, give very qualitative estimations of binding affinities through e.g. the MM/PBSA method,[100] and no indication of binding and unbinding paths. Hence, we performed metadynamics simulations on the best of the models we built, based on an X-ray GluCl template with 38% sequence identity,[15] to study the binding and unbinding of GABA to the orthosteric binding site in the wild-type and in two mutants, that were experimentally unresponsive.[32,66] Because sampling the entire solvent is an unrealistic task within the time simulations can afford, we used a funnel restraining potential enclosing the binding site, as roughly identified by mutagenesis experiments and preliminary MD simulations. The neurotransmitter could freely move within the funnel, which was positioned as shown in Fig. 6. The funnel potential limited the exploration of the solvent to a small cylindrical volume, whose effects on the binding affinity could be corrected *a posteriori*.[101] The two CVs biased in the metadynamics were: (i) the position of the centre of mass of GABA along the funnel axis and (ii) the one along the radius orthogonal to such axis. Simulation times of the order of μs were needed for convergence and the AMBER ff12SB force field[102] was used.

In the wild-type RDL, first the GABA carboxylate group binds from the solvent to the positively charged Arg218, the initial point of attraction in the receptor, and then to Arg111, to which it remains pinned, swinging its amine tail to interact with Glu204 and, in the process, dragging the C-loop over the orthosteric binding site. GABA positioned in

between Glu204 and Arg111 is shown in Fig. 6. Mutating either Glu204 or Arg111 with the neutral alanine dramatically disrupts the free energy landscape, as evident in the projection of the free energy along the funnel axis in Fig. 6. A pre-binding minimum is also noticeable in the wild-type case, where GABA first binds to Arg111 before swinging its amine tail to interact to Glu204, surrounded by a cage of aromatic residues. The disruption by mutations is also clear from the energy landscape re-weighted[103] with respect to the distances between GABA charged moieties and the C_αs of the residues at position 104 and 111, also shown in Fig. 6. In the wild-type the two isoenergetic minima correspond to binding poses where the interaction of GABA with Glu204 was either direct or mediated by a water molecule. In the Arg111Ala-mutated receptor, the first part of the binding process was disrupted: the absence of Arg111 prevented GABA from finding its first stable anchoring point and entering the binding site with optimal orientation. Alternative binding interactions only gave rise to shallow free energy minima. In the Glu204Ala-mutated receptor, GABA carboxylate still bound to Arg218 and Arg111, as in the wild-type receptor, but the amine group could not form any persistent interaction due to the absence of Glu204, so the process could not be completed. The data for the mutated receptors are consistent with weak or no binding, when including the entropic cost of the funnel potential, in agreement with the mutagenesis experiments which recorded non-responsive channels.[32,66]

We also used metadynamics to assess the effect of mutating a potential *trans-cis* proline switch, which was experimentally suggested as crucial for the gating of the 5-HT_3 receptor.[56] This proline residue (Pro8*) is located in the M2-M3 loop (shown in Fig. 1), which is important for the transduction of the binding signal to the channel gate. Mutagenesis electrophysiology experiments established that if this specific proline was mutated with analogues that would disfavour the *trans-cis* isomerisation, the channel would not function.[56] The proposed idea of the *trans-cis* switch involved in the opening of the ion channel is intriguing, although still controversial;[104,105] more experimental and computational studies would be useful to assess it.

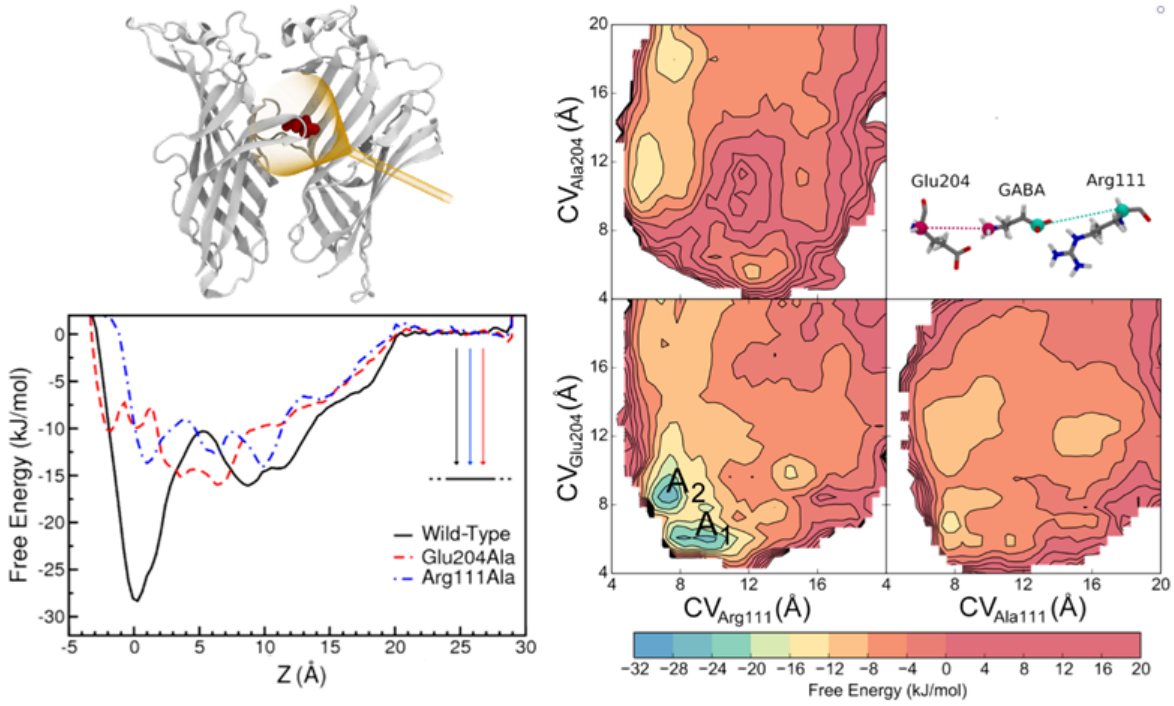


Figure 6: Top left panel: Location of the funnel restraining potential with respect to two adjacent subunits in the ECD of the RDL receptor; GABA is shown in red. Bottom left panel: GABA binding free energy projected onto the funnel axis for the wild-type RDL receptor and the Glu204Ala and Arg111Ala mutants. Right panels: GABA binding free energy as a function of the distances between GABA charged groups and the C_αs of the residues at positions 111 and 204 (as sketched in the top right corner in cyan and magenta) for the wild-type RDL receptor (bottom left), the Glu204Ala-mutant (top left), and the Arg111Ala-mutant (bottom right). [Adapted from Ref.[68]]

We calculated the free energy surface in water solution at room temperature for a simple dipeptide model of the switch for a number of mutants, as a function of two relevant torsional angles, as shown in Fig.7: ξ, which accounts for both the *trans-cis* prolyl peptide bond isomerisation and the coupled piramidalisation of the imide nitrogen, and ψ, which describes the orientation of the C-terminal amide. Convergence was reached in 16 ns. The *cis-trans* free energy differences relative to proline from metadynamics simulations and from electrophysiology experimental data (derived from the EC_{50}, for the functional wild-type and mutated channels) show a correlation coefficient of 0.98, indicative of a relationship between the *cis* and *trans* isomer populations and the receptor functional response. These calculations were done for simple models, which did not take into account the receptor environment, also because no experimental structure of 5-HT_3R was available. Simulations within the receptor are much more complex, requiring much larger systems and much longer simulation times for convergence. We have recently calculated, for the wild-type case with the model build from the X-ray structure of 5-HT_3R[17] shown in Fig. 1, how the proline isomerisation is affected by the protein environment (with a loss of symmetry in the free energy surface and a preferential direction for the isomerisation path), which in turns it affects, in particular at the level of the ring the negatively charged Asp residues at the top of the M2 helices, which act as a sort of selectivity filter for the incoming Na^+ ions.[106]

These data represent just a few examples of how metadynamics can be used to obtain additional information with respect to MD in the assessment of how mutations affect ligand-binding or switching mechanisms in pLGICs. There is potential to apply this technique to other processes involved in the chain of events leading to gating.[62]

Other protocols and enhanced sampling methods, from umbrella sampling to the string method, can also be used to obtain similar or complementary information, e.g. linked to conformational changes, ion translocations and binding kinetics in wild-type and mutated receptors.[107–109] In particular, the string method, which can be employed for computing transition pathways, free energy barriers and transition rates,[110,111] has been used to investigate

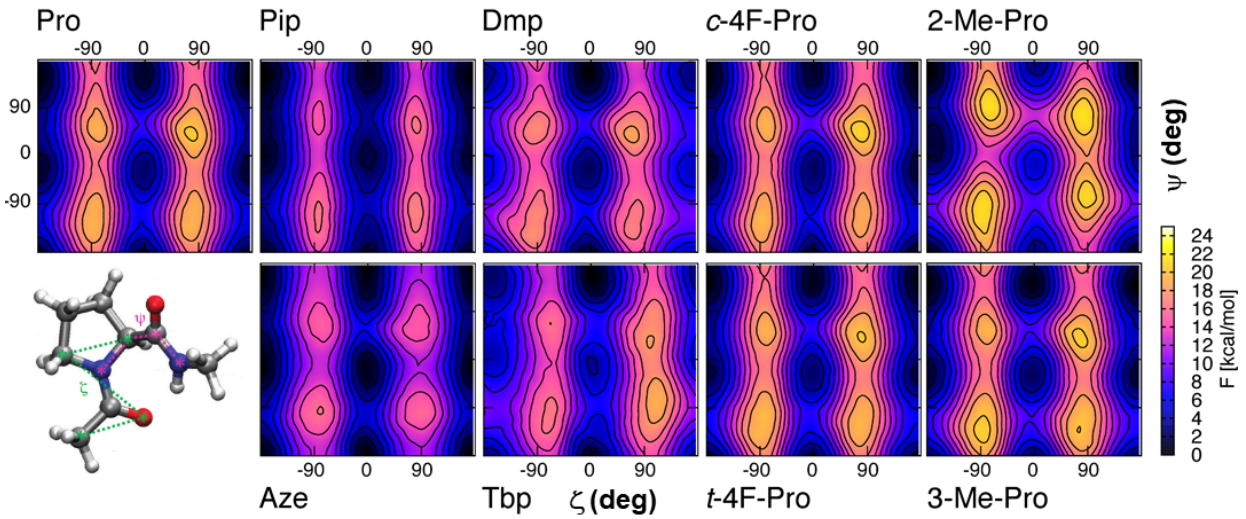


Figure 7: Free energy surfaces from metadynamics simulations, as a function of the angles ξ and ψ shown in the bottom left panel, for proline dipeptide (top right) and a number of mutants. [Adapted from Ref.[55]]

the activation of GLIC.[42,112]

With the increasing availability of structures in different functional states, enhanced sampling methods can play a crucial role in elucidating the dynamics between different functional states which is important to accurately describe the physiological behaviour of pLGICs.

Conclusions and Perspectives

A wealth of structural information from X-ray spectroscopy and cryo-electron microscopy and of functional data from mutagenesis electrophysiology experiments on pLGICs have become available. More data are expected to be generated in the near future: computational tools have a unique role to play in complementing experiments by providing insights at different levels of accuracy and resolution. Here, we reviewed a selection of the available computational techniques, which we, as well as others, have used to perform mutagenesis computer experiments in pLGICs with the goal to investigate specific processes, from ligand-

binding and modulation to residue isomerisation and channel hydration.

These techniques include first principles methods, which are useful to capture electronic structure details, especially important within the unnatural amino acid mutation strategy. An example is progressive fluorination of aromatic rings which disrupts aromaticity, weakening cation-π and hydrogen bond interactions; it would be difficult to describe these effects without explicitly accounting for the electronic degrees of freedom. First principles methods are also useful for fitting force-fields for unconventional residues and ligands. However, they are computationally costly and, hence, limited to simplified models and very reduced sampling times.

MD simulations with classical force-fields play a fundamental role in providing atomistic insights into partial or fairly complete pLGICs models, which are becoming more common and reliable as experimental structures with good resolution are being determined. In fact, the great majority of modelling work to investigate mutations in pLGICs is based on MD. With the support of ligand-protein docking methods and the guide of experiments, MD simulations are, for example, useful to study, at physiological or ambient conditions, ligated receptors, assessing how the ligand-protein interaction network changes when mutations are introduced. However, MD simulations also have shortcomings, due to the limited timescale they can sample.

In fact, activated processes in pLGICs need enhanced sampling methods to be meaningfully described: here we have focussed on two examples from metadynamics simulations.

The selection of methods we reviewed here is not unique and is based on those we have used in our work to investigate mutations in pLGICs. There are many other computational tools used in biomolecular simulations that can be useful for investigating pLGICs and their mutants, with on-going improvements and developments. For example, we did not discuss coarse grained (CG) methods, which can greatly simplify the models used in MD from all atoms to reduced number of sites, losing details but allowing more extensive sampling.[113] They have been applied in some studies of pLGICs: for example, elastic network models

were employed to explore the pathway of the gating transition in ELIC/GLIC,[38,114] and anaesthetic/pLGIC interactions were described with the MARTINI[115] CG force-field.[116]

Methods of different resolution can in principle be combined, not only at the QM/MM level already mentioned, but also at the MM/CG level, which have been recently tested for membrane proteins.[117] Mesoscopic and macroscopic levels may also be important to understand how pLGICs contribute to neuronal communication. While methods of different resolution are individually useful in the study of mutations in pLGICs as showed here, combining them in a comprehensive computational scheme, although very challenging, may improve the power of simulations in describing and predicting the working mechanisms of pLGICs and the influence that mutations have on them.

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Conflict of Interest

The Authors declare no conflict of interest.

Author Contributions

All authors discussed the content. AC and CMo wrote the paper. All authors revised and approved the paper.

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